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TECHNICAL NOTE

Measurements of cyclic AMP and cyclic GMP phosphodiesterase activity in isolated tubular segments

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The structural and functional heterogeneity of the renal tubular system is well recognized. To date however, the biochemical steps in the mechanism of action of hormones whose effects are mediated by cyclic 3′,5′-AMP (cAMP) have been studied primarily in the whole kidney or in major anatomic subdivisions (cortex, medulla, and papilla) of mammalian renal tissue [1, 2].

Recently, however, Imbert, Chabardes, and Morel introduced a micromethod for the assay of adenylate cyclase activity in specific nephron segments [3, 4] and demonstrated that adenylate cyclase stimulated by vasopressin (VP), parathyroid hormone, calcitonin, or isoproterenol is limited to well-defined segments of the renal tubular system [5].

Stimulation of adenylate cyclase, although important, is only the initial step in the sequence of intracellular biochemical events leading to the ultimate functional response of the tubule to a hormone. The rate of cAMP accumulation and the intracellular cAMP concentration are dependent not only on the rate of biosynthesis but also on the rate of hydrolysis by a specific enzyme, cyclic 3′,5′-AMP phosphodiesterase (cAMP-PDIE), and on the rate of efflux of cAMP from the cell [6]. Our previous studies [7] indicated that the distribution of enzymes of cAMP and cyclic 3′,5′-GMP (cGMP) metabolism were different in glomeruli compared to mixed cortical tubular fractions or to cortical slices, suggesting that the catabolism of cyclic nucleotides differs in specific substructures of the nephron.

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Cyclic nucleotide catabolism to date has not been studied in specific segments of the renal tubular system, except for a semiquantitative histochemical study of cAMP-PDIE in rabbit tissues, including kidney, which suggested the activity of this enzyme differed in various tubular segments [8]. In particular, high cAMP-PDIE activity was reported in the ascending limb of the loop of Henle, but little or no activity was reported in the collecting tubule. We have therefore developed a method for the determination of both cAMP-PDIE and cGMP phosphodiesterase (cGMP-PDIE) activity in specific tubular segments microdissected from the rat kidney. In particular, we have applied this technique to measure the activity of these two enzymes in the medullary collecting tubule (MCT) and the medullary thick ascending limb of the loop of Henle (MAL), two nephron segments recently shown to be major sites of VP-sensitive adenylate cyclase activity [3, 5, 9] and responding to VP and cAMP by changes in water permeability [10–13] or solute transport [14].

Methods and results

Adult male Sprague-Dawley rats (body wt, 200 to 250 g) were allowed free access to both food and water prior to sacrifice. Outer medullary tissue was prepared for microdissection by a method described by Imbert and Morel et al [3, 4] with several modifications. Briefly, while the rats were under light pentobarbital anesthesia (6 mg/100 g of body wt), the aorta directly above the left renal artery was clamped and the left kidney perfused with 15 ml of heparinized (20 USP units/ml) "*collagenase medium*" (for composition, see below) at a rate of 1.5 ml/min. Corticomedullary tissue slices were incubated in this medium for 30 min at 35° C, then washed thoroughly by rinsing in "*microdissection medium*"

Table 1. Adenylate cyclase activity sensitive to [8-Arginine]-vasopressin (AVP) and salmon calcitonin (SCT), in rat medullary thick ascending limb of Henle's loop (MAL) and in medullary collecting tubule (MCT)[a]

	MAL	*P*	MCT	*P*
Basal	12.4 ± 1.2 (5)		31.7 ± 5.2 (5)	
AVP (10^{-6} M)	322.3 ± 23.0 (4)	<0.001	366.0 ± 49.0 (5)	<0.001
SCT (0.1 μg/ml)	160.2 ± 23.9 (5)	<0.001	30.0 ± 4.8 (4)	NS

[a] Values for specific activity of adenylate cyclase (mean ± SEM) are expressed in fmoles/30 min/mm of tubule length. Numbers in parentheses denote the number of samples. *P* values are for significance of difference between basal and hormone-stimulated activities (group *t* test).

Table 2. Effect of freezing and thawing on cAMP-phosphodiesterase (cAMP-PDIE) activity in medullary thick ascending limb of Henle's loop (MAL) and medullary collecting tubule (MCT)[a]

	MAL	*P*	MCT	*P*
Nonfrozen	8.7 ± 0.8 (5)		5.0 ± 1.0 (4)	
Frozen	16.9 ± 2.6 (5)	<0.02	19.8 ± 1.1 (5)	<0.001

[a] Values for cAMP-PDIE activity (mean ± SEM) are expressed in fmoles/min/mm of tubule length. Numbers in parentheses denote the number of samples. *P* values are for significance of difference between frozen and nonfrozen activities (group *t* test).

(137 mM sodium chloride, 5 mM potassium chloride, 0.8 mM magnesium sulfate, 0.33 mM dibasic sodium phosphate, 0.44 mM monobasic potassium phosphate, 1.0 mM magnesium chloride, 10 mM Tris hydrochloric acid, 0.25 mM calcium chloride; pH, 7.4) and transferred to Petri dishes for microdissection. Subsequent preparative procedures were performed at 0 to 4° C. (Composition of "*collagenase medium*" was identical to "*microdissection medium*" except that calcium chloride concentration was 1 mM (instead of 0.25 mM); and 0.075% collagenase (Type I, 125-250 U/mg; Worthington Biochem. Corp., Freehold, New Jersey), 0.1% hyaluronidase (Type I, Sigma Chem. Co., St. Louis, Missouri), and 0.1% bovine serum albumin was added.

Individual tubular segments (0.5 to 2.0 mm in length) were dissected under stereoscopic magnification with sharpened stainless steel needles and transferred in a small volume (less than 1 μl) of "*microdissection medium*" by micropipette onto concave bacteriologic slides. All segments were then photographed for subsequent length measurement [4, 9]. The identity of each segment established by visual inspection was confirmed from the photograph by distinct morphologic characteristics [3, 4]. In addition, adenylate cyclase responses to VP (10^{-6} M [8-Arg]-vasopressin) and to synthetic salmon calcitonin (SCT; 0.1 μg/ml) were tested in a preliminary experiment (Table 1). Segments identified as ascending limb of Henle's loop (MAL) contained adenylate cyclase sensitive to both VP and SCT, whereas segments of medullary collecting tubules (MCT) contained only VP-sensitive adenylate cyclase, an observation consistent with that of Morel, Chabardes, and Imbert-Teboul [5] on other species.

Tubular samples were "permeabilized" by aspirating off the excess microdissection medium and replacing with 0.5 μl of "*hypotonic medium*" (1 mM magnesium chloride, 0.25 mM ethylene-diamine-tetracetic acid (EDTA), 0.1% bovine serum albumin (wt/vol), and 1 mM Tris hydrochloric acid; pH, 7.4), followed by rapid freezing on dry ice. Omission of this freezing and thawing step results in a significant reduction in cAMP-PDIE activity in both MAL and MCT, as observed in a preliminary experiment (Table 2). A similar observation was made with respect to adenylate cyclase activity measurements by Morel et al [4] and was attributed to an inability of enzyme substrate to enter the cell unless permeabilized by freezing and thawing. All samples were stored overnight at −80° C prior to assay. No significant loss of enzyme activity occurred in either MCT or MAL stored overnight at −80° C when compared with samples assayed the same day (Table 3).

Cyclic AMP-PDIE and cyclic GMP-PDIE activities were assayed by incubating permeabilized tubular segments in 5 μl of a medium of the following composition: 10 mM magnesium sulfate, 0.1 mM EDTA; 50 mM Tris hydrochloric acid; pH, 8.0 (Mg-EDTA-Tris buffer). Final substrate concentration (either ^{3}H-cAMP or ^{3}H-cGMP; New England Nuclear Co., Boston, Massachusetts) was 10^{-6} M (3×10^5 cpm per reaction mixture). After addition of the incubation mixture to the tubule, the slide was covered with a second vaseline-coated slide, providing a waterproof reaction chamber, which was immersed in a water bath maintained at 37° C for the desired length of time. At the end of the incubation period, 50 μl of Mg-EDTA-Tris buffer was added to the reaction mixture, and the mixture was transferred by micropipette into glass test-tubes (12 × 75 mm); the slide was rinsed with another 50 μl of Mg-EDTA-Tris buffer, which was added to the test tube. The test tubes were immersed for 3 min in boiling water to terminate the enzyme reaction. Fifty microliters of 5′-nucleotidase (snake venom from

Table 3. Effect of 24-hr storage at −80° C on cAMP-phosphodiesterase (cAMP-PDIE) activity in medullary thick ascending limb of Henle's loop (MAL) and medullary collecting tubule (MCT)[a]

	MAL	*P*	MCT	*P*
Same day	13.0 ± 1.0 (12)		14.3 ± 0.9 (12)	
24-hr storage	14.3 ± 1.0 (12)	NS	14.3 ± 1.1 (12)	NS

[a] Values for cAMP-PDIE activity (mean ± SEM) are expressed in fmoles/min/mm of tubule length. Numbers in parentheses denote the number of samples. *P* values are for significance of difference between same day and 24-hr storage activites (group *t* test).

Crotalus atrox; 1 mg/ml) were added to each tube and incubated for a further 15 min at 37° C as in our previous studies [7, 15]. Nucleotides were separated from nucleosides on QAE-Sephadex columns [7, 15] with 3 ml of 20 mM ammonium formate (pH, 7.4). Approximately 95% recovery from QAE-Sephadex columns was achieved under present conditions, results similar to those reported by Wells et al [16]. A standard assay blank of 5 μl of the reaction mixture without tubular protein was included in every assay. For cAMP-PDIE assays, the blank value never exceeded 2% (6000 cpm) of the total cpm added, and for cGMP-PDIE, the blank value never exceeded 4% (12,000 cpm) of the total counts per minute added.

Under the present conditions, cAMP hydrolysis in both MAL and MCT was linearly proportional to time for at least 40 min (hydrolysis of about 15% of substrate) (Fig. 1), whereas hydrolysis of cGMP progressed linearly with time only up to 15 min (hydrolysis of about 10% substrate), in both segments (Fig. 1). Therefore, in subsequent experiments, an incubation time of 20 min was used for the cAMP-PDIE assay and 10 min for the cGMP-PDIE assay. Both cAMP-PDIE and cGMP-PDIE activities were directly proportional to tubular length both in MAL and MCT over the ranges 1.0 to 7.0 mm and 1.0 to 5.0 mm, respectively (Fig. 2). Samples consisting of a total of approximately 1 to 2 mm of tubule were assayed in all subsequent experiments. Assays were conducted under conditions when activities were at least 2 to 3 times higher than blank values.

The specific activities of cAMP-PDIE and cGMP-PDIE in MAL and MCT were compared in such a way that segments of MAL and MCT microdissected from the same animal were always assayed for cAMP-PDIE or cGMP-PDIE simultaneously in order to allow paired comparison (Table 4). The specific activity of cGMP-PDIE was significantly higher ($P < 0.001$; Student's *t* test) that was cAMP-PDIE activity in both MAL and MCT. The rate of both cAMP and cGMP hydrolysis was similar in MCT compared with MAL, expressed per millimeter of tubule length (Table 4a). To compare the specific activities of cAMP- and cGMP-PDIE in isolated nephron segments with activities measured on renal homogenates and tissue extracts, we also calculated the data relative to tubular protein (Table 4b). Because it was not feasible to measure both enzyme activity and protein content on the same sample, such values could only be calculated indirectly from the actual tubular length, and a mean value for protein content for each segment, determined in separate experiments. Protein content was determined by a micromodification [17] of the micromethod of Lowry et al [18]; both samples and standards contained 1% sodium dodecyl sulfate (SDS). (SDS does not interfere with the protein determination [19].) The mean protein content of MCT (0.11 ± [SEM] 0.01 μg/min; $N = 24$) was simi-

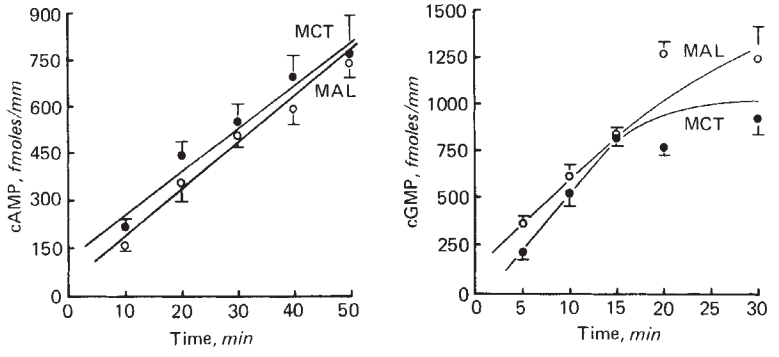

Fig. 1. *Dependence of cAMP* (left panel) *and cGMP* (right panel) *hydrolysis on the time of incubation in medullary collecting tubule* (MCT; - ● - ● -) *and in medullary ascending limb* (MAL; - ○ - ○ -). Each point represents mean ± SEM of four to six samples. For details see text.

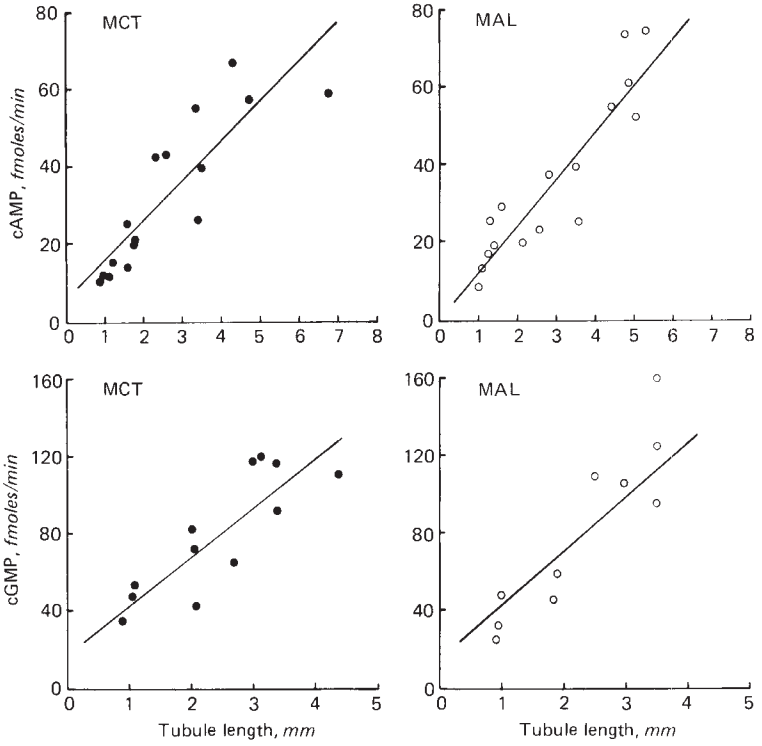

Fig. 2. *Dependence of cAMP-phosphodiesterase* (upper panels) *and cGMP-phosphodiesterase* (lower panels) *activity on tubular length in the incubation mixture. Left hand panels* represent medullary collecting tubule (MCT), and *right hand panels* represent medullary ascending limb (MAL). Regression lines were fitted by the method of least squares. For details see text.

lar to that of MAL (0.13 ± [SEM] 0.01 μg/mm; N = 35). When expressed per unit of protein, only minor differences in specific activities of either cAMP-PDIE or cGMP-PDIE existed between MCT and MAL (Table 4b).

Both cAMP-PDIE and cGMP-PDIE activities (Fig. 3) were almost completely inhibited by the addition of 5×10^{-4} M 1-methyl-3-isobutylxanthine (MIX)—a known phosphodiesterase inhibitor [1, 7, 13]—in both segments. A concentration of 5×10^{-5} M MIX caused a partial inhibition of both cAMP-PDIE and cGMP-PDIE in MAL and MCT.

Discussion

Results of the present studies demonstrate that the rate of cAMP and cGMP hydrolysis can be measured in specific microdissected segments of the renal tubule. Although there are probably several cyclic nucleotide phosphodiesterases in the kidney [20], this investigation was confined to one substrate concentration (10^{-6} M), which reflects predominantly, but not exclusively, the so called low K_m-high affinity enzyme activity [21]. cGMP-PDIE activity is higher than cAMP-PDIE is, in both MCT and MAL. Both cGMP and cAMP-PDIE activities

Table 4. Specific activities of cAMP-PDIE and cGMP-PDIE in rat medullary thick ascending limb of Henle's loop (MAL) or in medullary collecting tubule (MCT)[a]

	N	MCT	MAL	P^b	P^c
(a) fmoles/min/mm tubule length					
cAMP-PDIE	(14)	16.9 ± 9.9[a]	16.8 ± 0.7	NS	NS
cGMP-PDIE	(5)	52.6 ± 5.8	52.0 ± 6.0	NS	NS
(b) fmoles/min/μg protein					
cAMP-PDIE	(14)	153.8 ± 8.7[a]	129.7 ± 5.5	<0.05	<0.025
cGMP-PDIE	(5)	478.8 ± 53.6	400.4 ± 46.8	NS	<0.025

[a] Numbers in parentheses (N) denote the number of animals; value of each animal is the mean of three to five samples. Values for both cAMP-PDIE and cGMP-PDIE activities (mean ± SEM) are expressed in fmoles/min/mm of tubule length (*a*) and fmoles/min/μg of protein (*b*).

[b] Significance of difference between MCT and MAL (group t test)

[c] Significance of difference between MCT and MAL (paired t test)

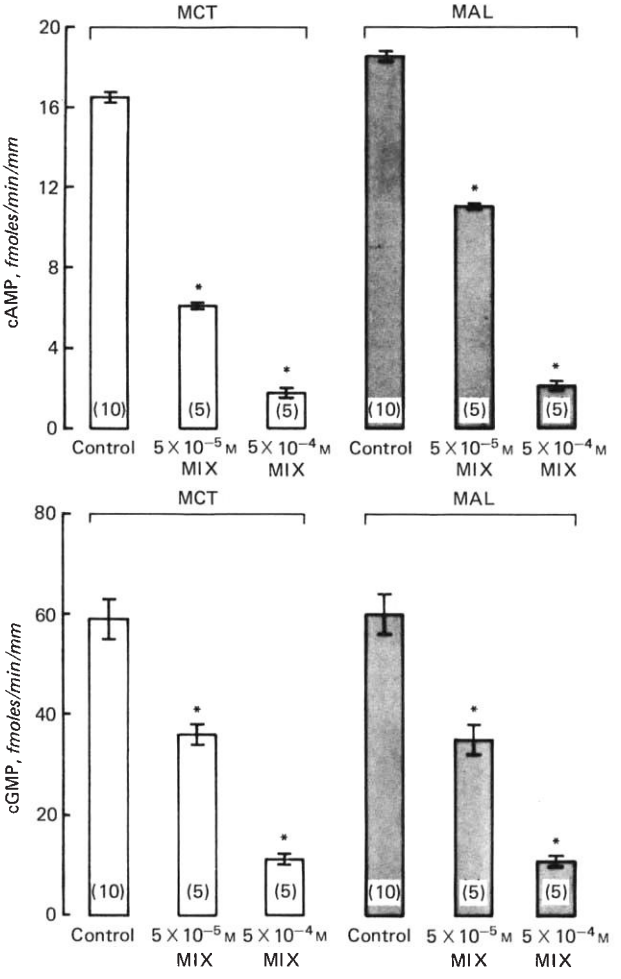

Fig. 3. *Inhibitory effect of 1-methyl-3-isobutylxanthine (MIX) on cAMP-phosphodiesterase* (top panel) *and cGMP-phosphodiesterase* (bottom panel) *activities.* Columns represent mean ± SEM; numbers in parentheses at the bottom of columns denote number of samples. *Asterisk* (*) denotes values significantly different ($P < 0.05$ or higher levels of significance; t test) from the corresponding controls.

are similar in these two segments. This observation of similar cAMP-PDIE activities in MCT and MAL differs from a previously reported histochemical study on the rabbit kidney [8] in which higher activity was reportedly present in the MAL. In that study, however, in addition to the species difference, a high (10^{-3} M) concentration of cAMP was used, and evaluation of PDIE activity was only semiquantitative.

The method we have developed is essentially a scaled-down version of the standard macroassay for phosphodiesterase used frequently both in our and other laboratories for determinations in tissue homogenates and fractions [7, 14, 15, 19]. A number of dissimilarities do exist, however. For example, technical limitations make it impossible for these small tubular fragments to be homogenized, and therefore, segments are made permeable to enzyme substrate by a process of freezing and thawing in a hypotonic solution [4], which in effect causes the cells to "disintegrate." The tubules after this treatment are essentially equivalent to a "crude homogenate." The necessity for this procedure, originally designed for measuring adenylate cyclase activity [3, 4], and later for determination of ATPase activity in single segments of the nephron [22], is demonstrated in Table 2, in which untreated tubular samples show significantly lower PDIE activity. It cannot be totally excluded that disintegrated tubular cells from different segments of the nephron may be differentially permeable to substrate. However, this would be extremely difficult to test directly. Under these conditions, it is perhaps more important to ensure that both segments are treated identically. It is unlikely that freezing and thawing directly effects phosphodiesterase activity. Our laboratory data for cortical homogenates showed that freezing and thawing had no effect on either cAMP- or cGMP-PDIE activity when compared with fresh preparations (unpublished observations).

Specific activities have generally been expressed relative to tubular length, as was done in previous studies in which adenylate cyclase [3, 4] and ATPase [22] activities were measured in isolated nephron segments. Alternatively, expression of specific enzyme activities relative to tubular protein content (calculated from tubule length and mean protein content for a given segment) may be of value when comparing specific activities of cAMP-PDIE and cGMP-PDIE of microdissected tubules with other preparations such as homogenates, and possibly in comparing activities of two segments with markedly different protein content. But because the mean protein content of MCT did not differ markedly from that of MAL, specific activities expressed either per tubule length or per tubule protein were similar for these two segments (Table 4, a and b). In addition, we find that the specific activities of both cAMP-PDIE and cGMP-PDIE in MCT and MAL compare favorably with the activities of these two enzymes measured in a mixed tubular suspension from rat renal cortex [7].

In contrast to the cortex, the specific activity of cGMP-PDIE is higher than is cAMP-PDIE activity, which may be compatible with reports that cGMP metabolism [23, 24], including breakdown [23], is more active in medulla and papilla than it is in the cortical zone of the rat kidney.

Our data would suggest then, that in the normal rat kidney, cAMP formation in response to VP does not appear to be either offset or favored by a differential rate of cAMP catabolism in the MCT compared with the MAL. VP-sensitive cAMP synthesis has been reported to be impaired in renal tubular disorders, such as the urinary concentrating defect seen in Brattleboro rats with diabetes insipidus [25], or in experimental uremia in rabbits [26], or in mice with hereditary nephrogenic diabetes insipidus [27]. The importance of measuring cAMP catabolism is illustrated by our recent finding that abnormal cAMP catabolism may play a key role in the inability of MCT to increase cAMP levels in response to VP [27]. We observed that in MCT from mice with nephrogenic diabetes insipidus, cAMP-PDIE activity is abnormally high and that VP responsiveness (in terms of cAMP accumulation) could be partially restored by treatment with a phosphodiesterase inhibitor MIX [27]. Thus, use of the methodology outlined in this communication in studying other models of renal tubular disorders may similarly provide new insights into the pathogenesis of anomalous responsiveness of renal tubules to VP [25, 26, 27] and to other hormonal agents [28] whose actions are mediated by cAMP.

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